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Rescue of AAV by Antibody-induced Fas-mediated Apoptosis from Viral DNA Integrated in HeLa Chromosome

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Adenoassociated virus (AAV) provirus in latently infected cells is rescued by superinfection with adenovirus. We examined helper-independent rescue of AAV by Fas-mediated apoptosis from the HeLa lines with AAV DNA integrated in chromosome (HeLa/AAV). In HeLa/AAV, anti-Fas antibody was found to induce low-level production of AAV virions, as detected by the presence of AAV DNA protected from DNase digestion. The antibody induced higher virion production in Fas-enriched HeLa/AAV, which was generated by transfecting HeLa/AAV with an expression plasmid for Fas, than in HeLa/AAV, and the rescued virions were shown to be infectious as assayed along with adenovirus. The antibody also induced apoptotic DNA fragmentation, as detected by staining intracellular fragmented DNA and electrophoresis, in HeLa/AAV and, more markedly, in Fas-enriched HeLa/AAV. Furthermore, an inhibitor for caspase-8 suppressed both the AAV virion production and the DNA fragmentation. Thus, the integrated AAV DNA is likely to be induced to initiate a low level of replication when Fas-mediated apoptosis is activated in HeLa cells.

Key Words: AAV provirus; rescue by apoptosis.

INTRODUCTION

Adenoassociated virus (AAV) type 2, a human parvovirus, is a nonenveloped small virus with a genome of 4.7-kb single-stranded linear DNA (Berns and Bohenzky, 1987; Srivastava *et al.,* 1983). Optimal propagation of AAV requires coinfection with a helper virus, such as adenovirus (Carter *et al.,* 1979; Richardson and Westphal, 1981; West *et al.,* 1987), herpes simplex virus (Buller *et al.,* 1981; Mishra and Rose, 1990; Weindler and Heilbronn, 1991), and human cytomegalovirus (Georg-Fries *et al.,* 1984; McPherson *et al.,* 1985). Without a helper virus the AAV genome is integrated into the host chromosomal DNA in a site-specific manner and is maintained as a latent provirus (Berns and Giraud, 1996). When a latently infected cell is superinfected with a helper virus, the integrated AAV is induced to replicate (Handa *et al.,* 1977; Cheung *et al.,* 1980). A low level of AAV replication is induced by irradiation of the latently infected cells with UV (Yalkinoglu *et al.,* 1988), or exposure of cells with genotoxic chemical carcinogens prior to infection with AAV (Yakobson *et al.,* 1987).

Apoptosis is the programmed cell death that occurs through multiple pathways (Nagata, 1997). In Fas-mediated apoptosis, Fas-ligand's binding to Fas (a member of the TNF receptor family or a type I-membrane protein) at the cell surface initiates a series of molecular events leading to cell death (Itoh *et al.,* 1991; Suda *et al.,* 1993; Itoh and Nagata, 1993; Nagata and Golstein, 1995). The binding induces trimerization of Fas, and its trimerized cytoplasmic region sends apoptosis signals. After the trimerization, the recruited and activated caspase-8 triggers the ICE protease cascade (Boldin *et al.,* 1996; Nagata, 1997) and finally activates caspase-3, resulting in activation of DNase (CAD), which digests chromosomal DNA into fragments (Enari *et al.,* 1998; Tang and Kidd, 1998; Inohara *et al.,* 1999; McIlroy *et al.,* 1999). Without Fas-ligand, the apoptosis can also be induced by cross-linking of Fas with agonistic anti-Fas antibodies (Alnemri *et al.,* 1996).

In this study, to test the hypothesis that AAV provirus has a system to escape from host cells dying of apoptosis, we examined the rescue of AAV by Fas-mediated apoptosis, using two newly established HeLa cell lines with AAV DNA in their chromosomes. It was found that anti-Fas antibody caused both production of infectious AAV virions and apoptotic DNA fragmentation in the HeLa cells. An inhibitor for caspase-8, which suppressed the DNA fragmentation, completely inhibited the rescue of the AAV. The results show that, from the AAV DNA integrated in the human chromosome, a low level of AAV replication can occur in the apoptotic cells without a helper virus.

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RESULTS AND DISCUSSION

HeLa cells with AAV DNA integrated in chromosome (HeLa/AAV)

To test our hypothesis, we attempted to prepare HeLa cell clones that contain the complete AAV genome integrated in cell chromosome (HeLa/AAV). HeLa cells were transfected with pUC19-AAV (7.1 kilobase pairs (kb long) that had been linearized by digestion with *Sca*I cleaving pUC19 sequence at a single site, together with an expression plasmid for selectable blasticidin-resistant gene, pSV-bls. One hundred three clones of blasticidin-resistant HeLa were obtained and examined for the presence of AAV DNA in the cellular DNA by agarose gel electrophoresis after amplification of DNA fragments encoding viral capsid proteins (VP-1 to -3) by PCR. Ten clones positive for the DNA fragment were selected and were further screened for the presence of rescuable AAV DNA in them. The cells were inoculated with adenovirus type 2 (Ad) at a multiplicity of infection (m.o.i.) of 100, and at 48 h after infection levels of AAV DNA were measured by semiquantitative PCR with the supernatant fraction of the modified Hirt's extraction (Arad, 1998), which presumably contains AAV virion DNA. Two clones that produced the highest level of AAV DNA, HeLa/AAV-53 and -107, were selected for further characterization regarding production of infectious virions and state of viral DNA in the cells.

HeLa/AAV-53 or -107 was found to produce infectious AAV virions after infection with Ad. HeLa/AAV-53 or -107 (5×10^6 cells) in a 10-cm plate was infected with Ad at an m.o.i. of 30 and incubated for 72 h. Cells were harvested, resuspended with 1 ml of PBS, freeze-thawed five times, incubated at 60°C for 30 min to inactivate Ad, and then centrifuged at 15,000 *g* for 5 min to obtain the supernatant. Fresh HeLa cells were inoculated with the supernatant, which was digested with DNase to eliminate free-DNA, together with fresh Ad. Forty-eight hours later, presence of AAV capsid proteins in cell lysate was examined by immunoblotting (Fig. 1A). AAV capsid proteins were expressed in HeLa cells inoculated with the supernatant, indicating that the supernatant contained infectious AAV. Furthermore, AAV virions, icosahedral particles with a diameter of 20 nm, was found in the supernatant by electron microscopy (data not presented). Thus, it was concluded that, similar to the cells latently infected with AAV, HeLa/AAV-53 and -107 contained complete AAV DNA that can be rescued by Ad infection.

HeLa/AAV-53 and -107 were examined for the state of AAV DNA by Southern blotting using total cellular DNA and the probe for AAV DNA (Fig. 2). The DNA samples not digested with any restriction enzymes showed the signals near the top of the separating gel, indicating that AAV DNA migrated with high molecular weight cellular DNA (Fig. 2A). Signals for DNA with a size of free AAV

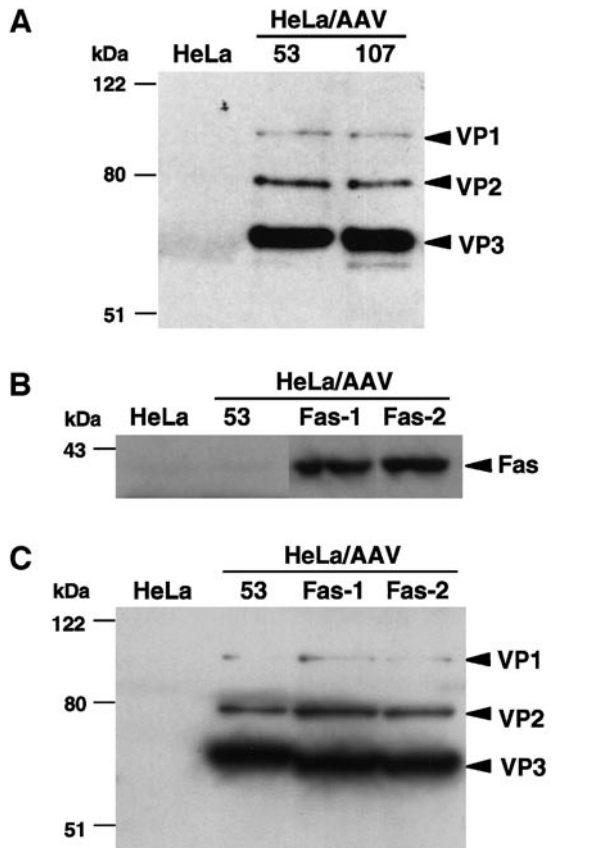

FIG. 1. Detection of AAV capsid proteins and Fas by immunoblotting. (A) AAV capsid proteins, VP-1 to -3, expressed in HeLa inoculated with AAV virions rescued by Ad superinfection. HeLa cells were inoculated with lysates from Ad-infected HeLa/AAV-53 and -107, together with Ad. Cellular lysate obtained 48 h later was electrophoresed in a 10% polyacrylamide gel and the proteins were transferred to a nylon membrane. Capsid proteins were detected with anti-AAV capsid antibody. (B) Detection of Fas overexpressed in HeLa/AAV/Fas-1 and Fas-2. Cellular lysates were analyzed by immunoblotting with anti-Fas antiserum. (C) AAV capsid proteins expressed in HeLa/AAV clones after Ad infection. The cells were infected with Ad at an m.o.i. of 50. Cellular lysates were obtained at 48 h (HeLa and HeLa/AAV-53) or at 72 h (HeLa/AAV/Fas-1 and -2) postinfection and analyzed by immunoblotting with anti-AAV capsid antibody. Lines 53 and 107 represent HeLa/AAV clones and Fas-1 and Fas-2 represent HeLa/AAV/Fas clones.

DNA or pUC19-AAV were not detected even after prolonged exposure (data not presented). Therefore, these results suggest that, in HeLa/AAV-53 and -107, AAV DNA is integrated within cell DNA.

Cleavage with *Xba*I, which does not cut double-stranded AAV DNA and pUC19-AAV, gave rise to a broad and intense signal band in HeLa/AAV-53 DNA and a clear single band in HeLa/AAV-107 DNA (Fig. 2B). These DNA fragments were 11-kb long, which were longer than AAV DNA (4.7 kb) or pUC19-AAV. The intensity of the band in HeLa/AAV-53 DNA suggests that this band contained a mixture of two to three copies of AAV DNA flanked by nonviral sequences. When the membrane blotted with the *Xba*I-digested DNA samples was reprobed with the AAVSI BamHI segment (Lamartina *et al.,*

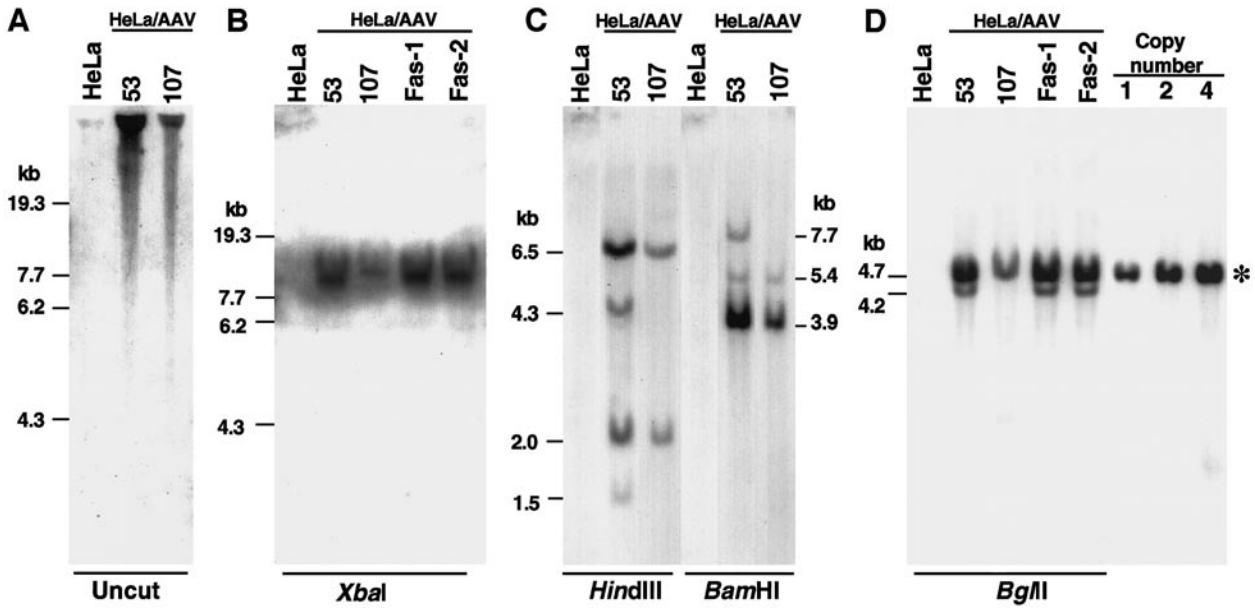

FIG. 2. Southern blotting analyses of restriction DNA fragments from HeLa/AAV and HeLa/AAV/Fas. Total cellular DNA was extracted from the cells indicated. Eighteen micrograms of DNA, undigested or digested by restriction enzymes, was electrophoresed through a 0.8% agarose gel and immobilized on a nylon membrane. (A) Undigested DNA. (B) DNA cleaved with *Xba*I (no cut enzyme for double-stranded AAV DNA and pUC19-AAV). (C) DNA cleaved with *Hin*dIII or *Bam*HI (one cut enzymes). (D) DNA cleaved with *Bgl*II, which release AAV DNA from pUC19-AAV. For comparison, *Bgl*II-digested pUC19-AAV, which corresponded to one, two, and four genome copies of double-stranded form per cell, was electrophoresed in the presence of *Bgl*II-digested normal HeLa DNA. Probes were prepared by labeling of AAV DNA with ^{32}P by random primer labeling kit (Rediprime II DNA labeling system; Amersham Pharmacia Biotech). The double-stranded AAV DNA is indicated by an asterisk. Lines 53 and 107 represent HeLa/AAV clones and Fas-1 and Fas-2 represent HeLa/AAV/Fas clones.

2000), the probe hybridized with a DNA fragment different from the 11-kb DNA (data not shown), which indicate that, in HeLa/AAV-53 and -107 cells, AAV DNA was not integrated in the AAVS1 region.

Cleavage with *Hin*dIII, which cleaves AAV DNA once (also cut pUC19-AAV once), generated four and two bands, respectively (Fig. 2C). Interestingly, bands of 6.5 and 2.0 kb were found in both HeLa/AAV-53 and -107 DNA and were more intense in HeLa/AAV-53 than in -107. Cleavage with another one-cut enzyme, *Bam*HI (also one-cut for pUC19-AAV), resulted in three bands in HeLa/AAV-53 and in two bands in HeLa/AAV-107. The bands of 5.4 and 3.9 kb were also found in both HeLa/AAV-53 and -107 DNAs and were more intense in HeLa/AAV-53 than in -107 (Fig. 2C). When the membrane blotted with *Hin*dIII- or *Bam*HI-cleaved DNA samples was rehybridized with the probe for pUC DNA, the bands were found at the same positions for those generated by the AAV-probe (data not shown). These results, along with the data obtained with the undigested DNA (Fig. 2A) and *Xba*I-digested samples (Fig. 2B), indicate that the AAV DNA flanked by pUC sequences is integrated, possibly at a similar site in cell DNA, in HeLa/AAV-53 and -107.

The integrated AAV DNA appeared to be released from high molecular weight cellular DNA by digestion with *Bgl*II, which can excise AAV DNA from pUC19-AAV (Fig. 2D). The digestion generated a 4.7-kb band corresponding to the complete AAV DNA with both HeLa/AAV-53 and -107 DNA, and with the former an additional less dense 4.2-kb band. Also, electrophoresed together for comparison were *Bgl*II-digested mixtures of pUC19-AAV and normal HeLa DNA (corresponding to one, two, and four copies of double-stranded AAV DNA per cell). Comparison of the intensities of the 4.7-kb bands indicates that HeLa/AAV-53 and -107 are likely to have two and one copies of complete AAV DNA, respectively. HeLa/AAV-53 appears to have one copy of 4.2-kb incomplete AAV DNA, probably lacking *Bam*HI site and thus yielding 7.7-kb band after the digestion (Fig. 2C). In HeLa/AAV-53 duplication of the area around the integrated AAV may have occurred after the integration.

Although they were morphologically indistinguishable from the parental HeLa cells, two clones HeLa/AAV-53 and -107, which were shown to have the integrated AAV DNA that can be rescued by Ad superinfection, showed slightly reduced growth rate and lowered saturation densities as reported previously for HeLa cells latently infected with AAV (Walz and Schlehofer, 1998).

Production of AAV virion by anti-Fas antibody in HeLa/AAV

After exposure of HeLa/AAV-53 and -107 to anti-Fas antibody, AAV virions emerged in the cell extracts, as detected as AAV DNA that was protected from digestion with DNase (Fig. 3). An extract from the cells (2×10^5)

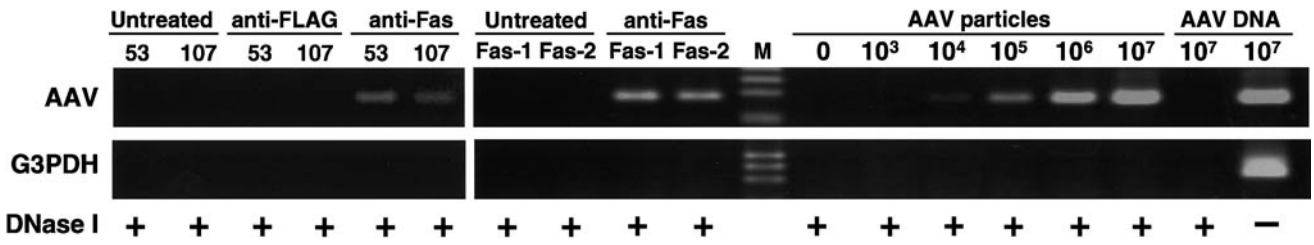

FIG. 3. Encapsidated AAV DNA produced in HeLa/AAV clones by stimulation with anti-Fas antibody. Cells cultured with or without antibodies (against Fas or FLAG) for 48 h were lysed and digested with DNase I to eliminate free-DNA. Then, encapsidated DNA, which was protected from the DNase digestion, was extracted and used as the template for the PCR to amplify AAV or G3PDH DNA. PCR products were electrophoresed in a 2% agarose gel and stained with ethidium bromide. For comparison, purified AAV virions were mixed with a cellular lysate of HeLa cells and processed similarly. Lines 53 and 107 represent HeLa/AAV clones and Fas-1 and Fas-2 represent HeLa/AAV/Fas clones.

which were cultured for 48 h with medium containing anti-Fas antibody (100 ng/ml) was digested with DNase, and then DNA for PCR to amplify AAV DNA was extracted. When the mixture of 10^7 genome copies of cloned AAV DNA and cellular DNA extracted from 2×10^6 of normal HeLa cells were digested under the conditions used in this experiment, AAV DNA and cellular DNA encoding G3PDH gene were not amplified by the PCR (Fig. 3). When AAV virions more than 10^4 genome copies were used instead of the cloned AAV DNA, the AAV DNA fragment was amplified by the PCR, indicating that DNA in virions were protected against the DNase digestion (Fig. 3). The levels of the amplified DNA fragment were parallel with those of input virions (Fig. 2). The results show that AAV DNA protected from DNase digestion was encapsidated in virions. Thus, stimulation with anti-Fas antibody appeared to induce replication of integrated AAV DNA in HeLa cells. Response to anti-Fas antibody was more prominent in HeLa/AAV-53 than in -107. An attempt to stimulate the cells with antibody against FLAG (a polypeptide unrelated to Fas) did not induce the replication (Fig. 3). Approximately 10^4 to 10^5 virions or genome copies of the encapsidated AAV DNA were present in the extract from 2×10^5 cells from HeLa/AAV-53 or -107 that had been cultured in the presence of anti-Fas antibody.

Induction of AAV production by anti-Fas antibody in HeLa/AAV enriched with Fas (HeLa/AAV/Fas)

To test whether the AAV production depends on the level of Fas on the cell surface, we prepared HeLa/AAV enriched with Fas (HeLa/AAV/Fas) and examined its response to the antibody regarding AAV virion production.

HeLa/AAV-53 was transfected with the newly constructed expression plasmid for Fas, pCMV-Fas, which contains an expression unit for G418-resistant gene. Immunoblotting using anti-Fas antibody showed that, while the level of Fas in HeLa was low as previously reported (Muhlenbeck *et al.,* 1998), the two clones, designated HeLa/AAV/Fas-1 and -2, overexpressed Fas (Fig. 1B). These two HeLa/AAV/Fas clones were selected from 24 drug-resistant clones.

In Southern blotting analysis, the AAV DNA in *Xba*I- or *Bg*/II-digested cellular DNA of HeLa/AAV/Fas-1 and -2 was indistinguishable in size from that of the parental HeLa/AAV (Figs. 2B and 2D). Both HeLa/AAV/Fas clones expressed AAV capsid proteins upon infection with Ad (Fig. 1C), indicating complete AAV DNA was present in these cells. In HeLa/AAV/Fas, the cytopathic effects by Ad emerged slowly and the levels of AAV capsid proteins were low. The overexpression of Fas probably influences growth of Ad, due to yet unknown mechanisms.

Production of AAV virions was induced by anti-Fas antibody more efficiently in HeLa/AAV/Fas-1 and -2 than in the parental HeLa/AAV (Fig. 3). The dependency on the Fas level indicates that Fas was involved in the antibody-induced AAV virion production. Approximately 10^5 to 10^6 virions or genome copies of encapsidated AAV DNA were found to be present in the extract from 2×10^5 cells of HeLa/AAV/Fas-1 or -2 stimulated by anti-Fas antibody.

Infectious virions were found in the extract from HeLa/AAV/Fas-1 stimulated with anti-Fas antibody. The DNase-digested extract from the cells of 2×10^4 was inoculated to each of three wells containing 3×10^5 cells. Twenty-four hours later the HeLa cells were superinfected with Ad (m.o.i. 50) and further incubated for 72 h. The cells in the each well were harvested, resuspended in 200 μl of PBS, sonicated, and then centrifuged. DNA was extracted from the supernatant and used for the template of PCR to amplify AAV DNA (Fig. 4). The extracts from two of the three wells were positive for the amplified fragment of AAV DNA. In this experiment, the extract from the three wells inoculated with purified AAV virions of more than 10^5 genome copies were positive for the AAV DNA (Fig. 4). One of the three wells was positive when 10^4 genome copies were used for the inoculum. Therefore, the extract from 2×10^4 cells of HeLa/AAV/Fas-1 stimulated by anti-Fas antibody was estimated to contain 10^4 to 10^5 genome copies of virions. The number of virions is consistent with the number of the encapsidated AAV DNA copies.

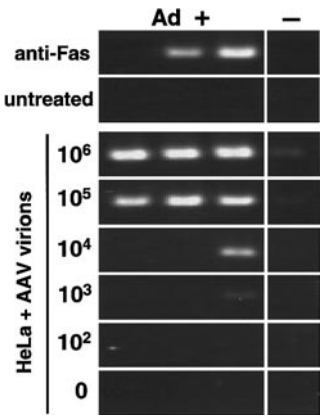

FIG. 4. Infectivity of the rescued AAV from HeLa/AAV/Fas-1 by anti-Fas antibody. The rescued AAV was inoculated to HeLa cells with or without Ad. For comparison, purified AAV virions were inoculated to HeLa similarly. Seventy-two hours later, the infected cells were lysed and incubated with DNase I. AAV DNA protected from the digestion was extracted from virions and was amplified by PCR. The amplified DNA was electrophoresed in a 2% agarose gel and stained with ethidium bromide.

Apoptotic DNA fragmentation caused by anti-Fas antibody

Anti-Fas antibody was shown to cause cell DNA fragmentation in HeLa/AAV and Fas-enriched HeLa/AAV, by TUNEL, which is a method to label the 3′ end of intracellular fragmented DNA for cytological detection and by agarose gel electrophoresis of extracted DNA. The cells were cultured in the presence of anti-Fas antibody (100 ng/ml) for 48 h and were then subjected to the two assays. After TUNEL staining, whereas DNA fragmentation was recognized in approximately 1% of the HeLa/AAV cells, it was recognized in 20 and 10% of HeLa/AAV/Fas-1 and -2 cells, respectively (Fig. 5A). These cytological observations were consistent with the electrophoresis data obtained with the extracted cellular DNA (Fig. 5B). Presence of fragmented chromosomal DNA was evident in both HeLa/AAV and HeLa/AAV/Fas cells, but clearly more prominent in the latter. The fact that HeLa/AAV/Fas was more sensitive to the antibody than HeLa/AAV appears to be evidence that the antibody-induced DNA fragmentation resulting from Fas-mediated apoptosis. It should be noted that the HeLa/AAV cells appeared to be significantly more sensitive to induction of apoptosis by anti-Fas antibody than the parental HeLa cells.

If we assume only the apoptotic cells recognized by TUNEL had been producing AAV virions, we could estimate their yield per cell from the data in Figs. 2 and 4A. In HeLa/AAV/Fas, the yield in 48 h would be approximately in the range of 50 to 100 virions per TUNEL-positive cell. The yields calculated for HeLa/AAV-53 and -107 fell into the lower part of the range. The data suggest that the level of the virion induction is dependent on the extent of Fas-mediated apoptosis in HeLa cells.

Inhibition of AAV production and cell DNA fragmentation in Fas-enriched HeLa/AAV by caspase-8 inhibitor

Caspase-8 inhibitor, z-IETD-fmk (Martin *et al.,* 1998), was shown to inhibit anti-Fas antibody from inducing AAV virions and DNA fragmentation in HeLa/AAV/Fas-1. The cells were cultured in the growth medium containing the inhibitor (50 μM) for 60 min and then further incubated in the medium containing the inhibitor plus anti-Fas antibody for 48 h. The encapsidated AAV DNA, which would be induced by the antibody, was not detected in the extracts from the cells treated with the inhibitor (Fig. 6A). In the presence of the inhibitor, the antibody did not cause the DNA fragmentation (Fig. 6B). Thus, it was shown that activation of caspase-8, which triggers the caspase cascade in Fas-mediated apoptosis, is required for the DNA fragmentation and the induction of AAV in HeLa/AAV/Fas, indicating that the antibody induction of AAV production is indeed associated with the apoptosis.

In summary, we found that AAV can be rescued, albeit inefficiently, from viral DNA integrated in HeLa chromosome by the antibody-induced Fas-apoptosis. The results of this study support our hypothesis on the AAV's ability to escape from the programmed cell death, although it remains to be studied whether the AAV provirus can be rescued by apoptosis in natural latent infection.

It may be that, when the host cell is going to be excluded by apoptosis, the AAV provirus initiates replication without a helper virus. The helper-independent replication would contribute to survival of AAV *in vivo,* since the incidence of helper infection would not be very high for a cell with the latent AAV at the right time before cell death. The level of infectious AAV produced in the apoptotic cells in this study was much lower than that produced in the cells infected with Ad, suggesting that cells in the course of apoptosis do not contain enough cellular or viral factors that can be supplemented by Ad infection to support efficient AAV replication. Although the induced AAV level is low in apoptosis, it is probably sufficient for AAV to escape from the programmed cell death and infect surrounding cells.

It has been reported that the helper-independent AAV replication occurs in cells irradiated with UV or pretreatment with genotoxic chemicals (Yakinoglu *et al.,* 1988; Yakobson *et al.,* 1987). These stresses give damage directly on DNA at the beginning and the damage induces activation of ATM-kinase and p53, resulting in the cell growth arrest to repair the DNA damage or activation of the caspase cascade to kill the injured cells (Meyn, 1995; Banin *et al.,* 1998; Canman *et al.,* 1998). The cellular mechanisms for DNA repair are believed to be important for the cells' helper functions (Berns and Bohenzky, 1987; Zentilin *et al.,* 2001). However, the DNA repair system is considered not to be associated with Fas-mediated apoptosis. Thus, this is the first report of the helper-inde-

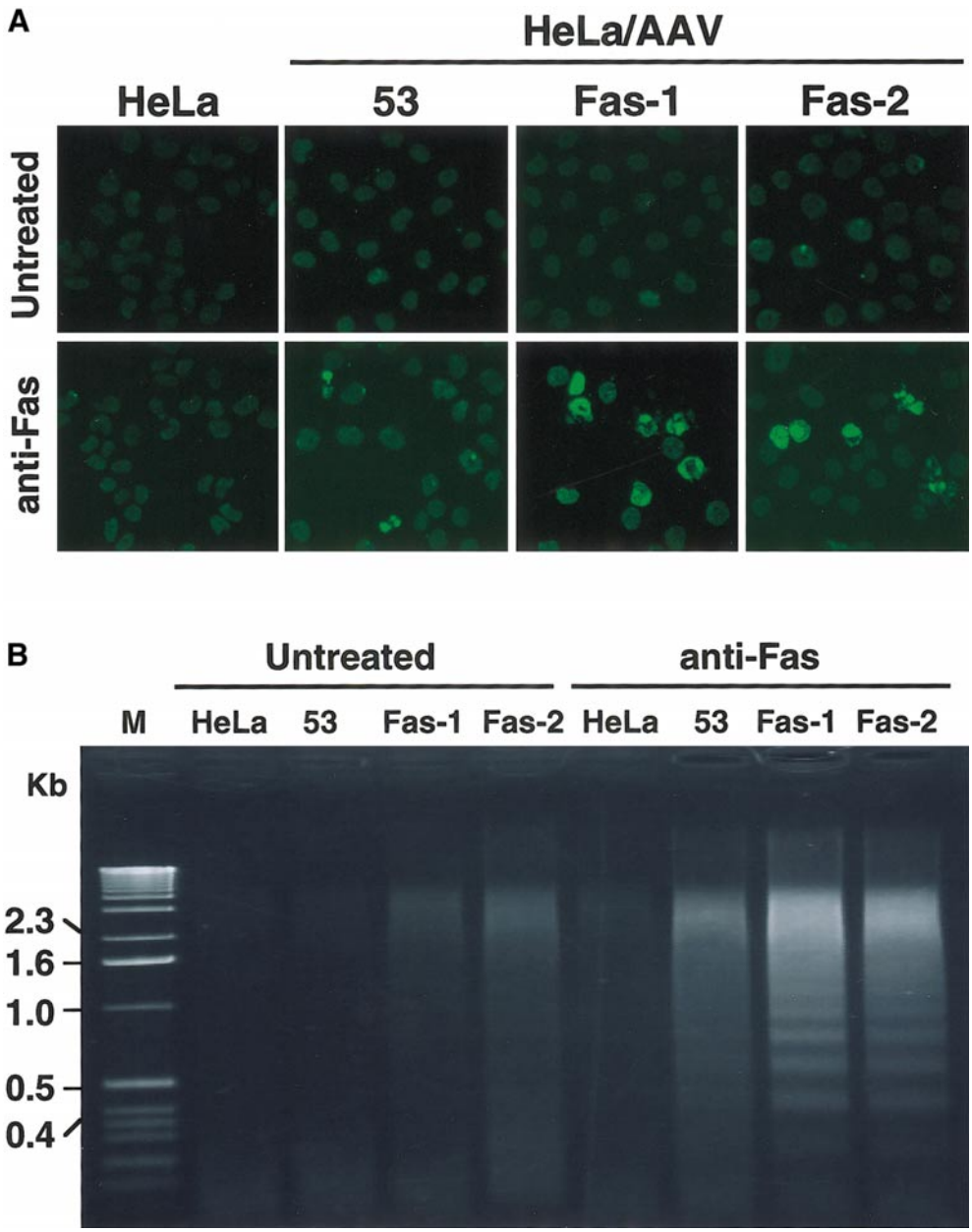

FIG. 5. Apoptotic DNA fragmentation in HeLa/AAV and HeLa/AAV/Fas clones cultured with or without anti-Fas antibody for 48 h. (A) The cells containing fragmented DNA were detectable by staining the 3′ ends of DNA by TUNEL method in the cultures without anti-Fas (top) and in those with anti-Fas (bottom). (B) Total DNA extracted from the cells was electrophoresed in a 1.2% agarose gel and stained with ethidium bromide. Fragmented DNA was recognizable as heterogeneous fast moving DNA. Line 53 represents HeLa/AAV clones and Fas-1 and Fas-2 represent HeLa/AAV/Fas clones.

pendent AAV replication without activation of DNA repair mechanism. It would be interesting to study how the integrated AAV DNA is activated to replicate, using the molecular events activated by the apoptosis.

MATERIALS AND METHODS

Plasmids

AAV2 complete genome excised from pAV1 (purchased from ATCC, no. ATCC37215) by *Bgl*II digestion was cloned at *Bgl*II site which was newly generated between two *Pvu*II sites of pUC19 by insertion of *Bgl*II linker. The resultant plasmid, pUC19-AAV, was used for transfection to produce HeLa cells containing AAV DNA integrated in chromosomes. pSV2-brs was purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan).

An expression plasmid for Fas, pCMV-Fas, was constructed by insertion of cDNA encoding Fas into pCMV-Tag4 (Stratagene, La Jolla, CA) at its *Eco*RI site. The cDNA for Fas in TG-1 (primary human fibroblast) cDNA

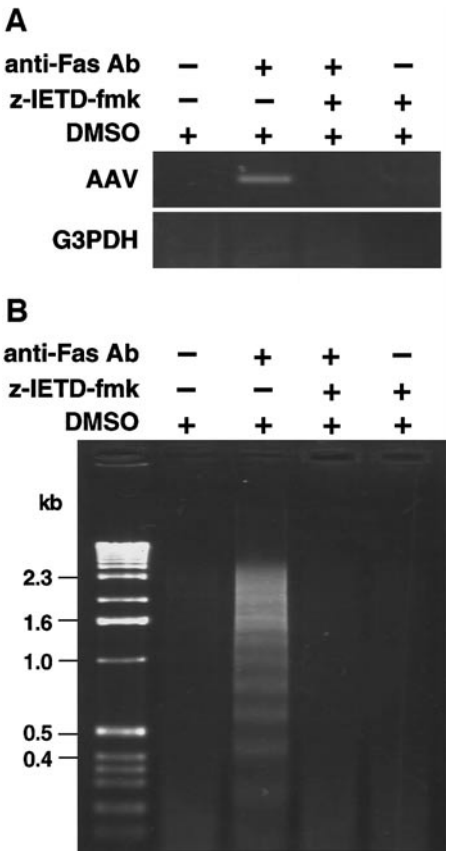

FIG. 6. Effect of z-IETD-fmk, a caspase-8 inhibitor, on induction of encapsidated AAV DNA (A) and apoptotic DNA fragmentation (B) in HeLa/AAV/Fas-1 (see Materials and Methods for details).

library was amplified by PCR using primers of 5′-TCAACAACCATGCTGGGCATCTGG and 5′-CTAGACCAAGCTTTGGATTTC. The nucleotide sequence of the amplified DNA matched that in the data base (GenBank Accession No. M67454).

Reagents

Anti-Fas mouse monoclonal antibody (CH-11) used for stimulation of HeLa/AAV cells and anti-Fas rabbit polyclonal antibodies (sc-714) used for immunoblotting were purchased from MBL Co., Ltd. (Nagoya, Japan) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Anti-FLAG antibody used for attempted stimulation was purchased from Sigma-Aldrich (St. Louis, MO). Caspase inhibitor, z-IETD-fmk, which inhibits activity of caspase-8, was purchased from Calbiochem-Novabiochem (La Jolla, CA). Restriction enzymes and DNase I was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

Transfection of DNA to HeLa cells

Cells used in this study were cultured in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and grown in 5% CO_2 at 37°C. Subconfluent culture of the cells in a 10-cm plate was transfected with 2 μg of plasmid DNA using Effectene (Qiagen GmbH, Hilden, Germany). After incubation for 48 h, the cells were passaged at a split ratio of 1 to 10 and cultured in the growth medium containing a selective drug, brasticidin (2 ng/ml) or G418 (500 μg/ml). Drug-resistant cell clones were obtained by three successive single colony isolations.

Immunoblotting

To detect AAV capsid proteins, cells were infected with adenovirus type 2 (Ad) at an m.o.i. of 50, incubated in growth medium for 48 to 72 h, suspended in the medium by a rubber policeman, and incubated at 60°C for 30 min. The cells were precipitated by a centrifugation of 3000 rpm for 10 min, resuspended in PBS, sonicated briefly, and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with an equal volume of 2× SDS sample buffer (SDS sample buffer: 153 mM Tris–HCl, pH 7.5, 4.9% SDS, 6.13% 2-mercapt ethanol, 24.5% glycerol, and 0.0025% bromphenol blue) and boiled for 5 min. To detect Fas, cells were collected by a centrifugation of 3000 rpm for 10 min, suspended in appropriate volume of SDS sample buffer, and boiled for 5 min.

The samples were electrophoresed in 10% SDS–PAGE and the proteins were transferred to nylon membrane, Hybond-P (Amersham Biosciences Inc., Uppsala, Sweden). After blocking with 5% skim milk, VP proteins of AAV were probed by anti-VP mouse monoclonal antibody (#65158, Progen GmbH, Heidelberg, Germany), and Fas was probed by anti-Fas rabbit antiserum, sc-714. Horseradish peroxidase conjugated anti-rabbit IgG goat antibodies (SC-2020, Santa Cruz Biotechnology, Inc.) and ECL Western Blotting Detection System (Amersham Biosciences Inc.) were used to detect mouse anti-AAV-VP or anti-Fas IgG.

Southern blotting

Cellular DNA was extracted and purified with Qiagen Genomic-tip system (Qiagen GmbH). Eighteen micrograms of DNA was digested with restriction enzymes, *Xba*I, *Hin*dIII, *Bam*HI, and *Bgl*II, electrophoresed on 0.8% agarose gel, and transferred to a nylon membrane (Hybond-XL, Amersham Biosciences, Inc.). ^{32}P-labeled probes were prepared by Rediprime II DNA labeling system (Amersham Biosciences Inc.).

Detection of AAV DNA protected from DNase I

Cells (2×10^5) in a 35-mm culture plate were incubated in growth medium containing anti-Fas antibody (100 ng/ml) for 48 h. When effect of caspase-8 inhibitor was examined, cells were incubated with growth medium containing z-IETD-fmk (50 μM) for 60 min, refed with growth medium containing z-IETD-fmk (50 μM) and anti-Fas antibody (100 ng/ml), and incubated for 48 h. Since z-IETD-fmk was suspended in dimethyl sulfoxide

(DMSO), control cells were incubated with growth medium containing DMSO (0.1%). The cells were scraped off from the plastic surface by a rubber policeman, collected by low speed centrifugation, resuspended in 200 μl of PBS containing $MgCl_2$ (10 mM), and then sonicated briefly. For comparison, normal HeLa cells (2×10^6) were mixed with serially diluted purified AAV virions (0 to 10^7 genome copies) or cloned AAV DNA (pUC19-AAV, 10^7 genome copies) and sonicated. The sample was diluted with 800 μl of PBS containing $MgCl_2$ (10 mM), mixed with 500 μl of diethylether with vortex mixer, and an upper layer was removed after a centrifugation of 12,000 rpm for 2 min. The diethylether extraction was done twice and the sample was centrifuged 10,000 rpm for 10 min. The supernatant and DNase I (1400 units) were incubated at 37°C overnight and received 50 μl of 0.5 M EDTA to stop digestion with DNase. DNA was extracted from the resultant DNase-digested sample by using QIAamp DNA extraction kit (Qiagen GmbH). Twenty microliters out of total 200 μl of final DNA sample in TE buffer was used for PCR to detect AAV DNA.

Titration of infectious AAV in cell extracts

Normal HeLa cells (3×10^5/well) in 24-well culture plate were incubated with 100 μl of the DNase-digested sample (described above) prepared from HeLa/AAV/Fas-1 or from normal HeLa plus purified AAV virions at 37°C for 2 h and further incubated for 24 h after addition of 1 ml of growth medium. Then, the cells were infected with Ad (m.o.i. 50) and incubated for 72 h. The cells were scraped off by a rubber policeman in the medium used for cultivation and collected by low-speed centrifugation after incubation at 60°C for 30 min to inactivate Ad. The cells were resuspended in 200 μl of PBS, sonicated briefly, and centrifuged at 3000 rpm for 10 min. DNA was extracted from the supernatant using QIAamp DNA extraction kit (Qiagen GmbH). Twenty microliters of total 200 μl of final DNA sample in TE buffer was used for PCR to detect AAV DNA.

PCR

The following primers were used to amplify AAV DNA: forward primer: 5′-GAACTGGGTGGCCGAGAAGG (AAV nucleotides of 401 to 420, numbering of the nucleotide are according to AF043303 in GenBank), reverse primer: 5′-CTCTGAATCAGTTTTTCGCG (676 to 657). The following primers were used to amplify a part of G3PDH gene: forward primer: 5′-ACCACCATGGAGAAGGCTGG, reverse primer: 5′-GGATGATGTTCTGGAGAGCC. PCR consisted of the initial heating at 94°C for 5 min, followed by 37 or 35 cycles of incubation at 94°C for 30 s and then at 64°C for 2 min, and followed by the final incubation at 72°C for 7 min. Thirty-seven cycles were used for detection of AAV DNA protected from DNase digestion or G3PDH DNA (Fig. 2). Thirty-five cycles were used for titration of infectious AAV (Fig. 5).

Detection of apoptosis

Based on fragmentation of cellular DNA, apoptosis was detected by TUNEL or agarose gel electrophoresis. Cells (2×10^5) in a slide chamber were cultured with growth medium containing anti-Fas antibody (100 ng/ml) for 24 h. The fragmented DNA was stained with In situ cell death detection kit (Roche Diagnostics Co. Ltd., Mannheim, Germany).

Cells (1×10^6) in a 6-cm culture plate were incubated with growth medium containing anti-Fas antibody (100 ng/ml) for 24 h. Otherwise, cells were incubated with growth medium containing z-IETD-fmk (50 μM) for 60 min, refed with growth medium containing z-IETD-fmk (50 μM) and anti-Fas antibody (100 ng/ml), and then incubated for 24 h. DNA extracted from the cells using QIAamp DNA extraction kit (Qiagen GmbH) was examined for the fragmentation by ApoAlert LM-PCR kit (Clontech Co. Ltd., Palo Alto, CA) and agarose gel (1.2%) electrophoresis.

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